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Conjugational Transfer of Gentamicin Resistance Plasmids Intra- and Interspecifically in *Staphylococcus aureus* and *Staphylococcus epidermidis*

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We have previously reported the transfer of gentamicin resistance (Gm^r) plasmids in a mixed culture inter- and intraspecifically between strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated at Michael Reese Hospital (Jaffe et al., Antimicrob. Agents Chemother. **21**:773–779, 1982). We have now shown that representatives of these plasmids were transferred between apparently nonlysogenic strains of *S. aureus* either in mixed culture in broth or by filter-mating on agar medium. The mechanism of transfer appeared to be conjugation. A transferable Gm^r plasmid (pSH8) mobilized or cotransferred a tetracycline R-plasmid and a chloramphenicol R-plasmid that were not independently transferable. The transfer of Gm^r plasmids was accompanied by a high incidence of deletion mutations with varied loss of plasmid resistance determinants and, with some mutants, loss of the ability to effect self-transfer. Restriction endonuclease digestion of pSH8 and its deletion mutants made it possible to assign the property of self-transfer to a specific segment of the pSH8 genome and provided the basis for a physical and genetic map of that plasmid. Similar Gm^r plasmids from *S. aureus* strains isolated in locations remote from Michael Reese Hospital had resistance determinants and transfer properties comparable to those of pSH8. Our observations provide evidence for the conjugal transfer of some staphylococcal plasmids, apparently independent of the presence of phage. This mechanism may be of significance in the intra- and interspecific dissemination of resistance to aminoglycosides and other antibiotics in *Staphylococcus* spp.

We have reported the recovery of five classes of closely related gentamicin resistance (Gm^r) plasmids from *Staphylococcus aureus* and *Staphylococcus epidermidis* (13, 14). Each plasmid class was defined by its spectrum of antibiotic resistance markers, molecular mass, and restriction endonuclease digestion pattern. Apparently identical Gm^r plasmids of each class were present in epidemiologically related isolates of *S. aureus* and *S. epidermidis* (35). Similar results have recently been reported by others (2). Evidence of identity between tetracycline resistance plasmids in isolates of *S. aureus* and *S. epidermidis* has also been published recently (10, 12, 34). These findings support the hypothesis that the transfer of plasmids between *S. aureus* and *S. epidermidis* occurs in nature.

Transduction has been favored as the principal mechanism of genetic exchange between *S. aureus* strains, including the transfer of R-plasmids between *S. aureus* strains in a mixed culture (8, 20, 25). Transfer was not detected between nonlysogenic *S. aureus* strains, but was observed when a generalized transducing phage was present in the transfer system. R-plasmids native to *S. epidermidis* have been transduced intraspecifically by phages native to that species (21, 28, 29). The interspecific transduction of plasmids between *S. epidermidis* and *S. aureus* has not been reported, although a chromosomal marker, novobiocin resistance, has been reported to have been transduced between these two species via an *S. epidermidis* phage (40).

To determine a plausible mechanism for the intra- and interspecific exchange of Gm^r plasmids implied by our observations, we investigated this phenomenon further. Detailed studies of one of our transferable plasmids showed that it encoded its own transfer by a conjugation-like mechanism and also mobilized and cotransferred otherwise nontransferable plasmids. A specific region of plasmid DNA responsible for

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TABLE 1. Strains of *S. aureus* and *S. epidermidis* used

Strain designation	Relevant properties	Source/reference
RN450	Nonlysogenic derivative of *S. aureus* NCTC 8325, cured of prophages ϕ11, ϕ12, and ϕ13, susceptible to antibiotics and EtBr	R. P. Novick (24)
SH6	Gm^r clinical isolate of *S. aureus*	This laboratory Former designation BeA (14)
SH7	Gm^r clinical isolate of *S. aureus*	This laboratory Former designation SiA (14)
SH8	Gm^r clinical isolate of *S. aureus*	This laboratory Former designation McA (14)
SH9	Gm^r clinical isolate of *S. aureus*	This laboratory Former designation LiA (14)
SH10	Gm^r clinical isolate of *S. aureus*	This laboratory Former designation WaA (14)
SH11	Gm^r clinical isolate of *S. epidermidis*	This laboratory Former designation RoE (14)
SH21	RN450 lysogenized with ϕ11	This laboratory
SH22	Erythromycin-resistant mutant of RN450	This laboratory
SH23	SH22 lysogenized with ϕ11	This laboratory
SH24	Derivative of RN450, resistant to streptomycin and rifampin	This laboratory
SH25	SH24 lysogenized with ϕ11	This laboratory
SH26	Mutant of SH21, streptomycin dependent and resistant to rifampin	This laboratory
SH28	*S. epidermidis*, wild type, susceptible to all antibiotics and plasmid free	This laboratory
SH29	Mutant of SH28, resistant to streptomycin and rifampin	This laboratory
80CR5*str*	Restrictionless strain of *S. aureus*, nonlysogenic, resistant to streptomycin	(7)

plasmid transferability was identified by genetic and physical mapping. Other investigators have recently reported the similar conjugal transfer of staphylococcal plasmids (B. A. Forbes and D. R. Schaberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H116, p. 132).

(These results were presented in part previously [Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 21st, Chicago, Ill., abstr. no. 199, 1981].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. In prior epidemiological studies, we divided staphylococcal Gm^r plasmids isolated at

TABLE 2. Plasmid designations, properties, and sources

Plasmid designation	Relevant plasmid-mediated antibiotic resistance[a]	Mol mass (Md)	Source/reference
pC22.1	Cm	3	Shaw and Brodsky (31)
pI258	Em, Pc (β-lactamase)	20	Peyru et al. (27)
pSH5	Tc	~3	This laboratory, native to *S. aureus* SH9 (Table 1)
pSH6	Gm, Km, Tm	12	Strain SH6 (Table 1)
pSH7	Gm, Km, Pm, Tm	27	Strain SH7 (Table 1)
pSH8	EtBr, Gm, Km, Pm, Tm	30	Strain SH8 (Table 1)
pSH8Δ1	EtBr, Km, Pm, Tm	26	Gm^s mutant of pSH8
pSH9	Gm, Km, Pc (β-lactamase), Pm, Tm	38	Strain SH9 (Table 1)
pSH9Δ1	Km, Pc (β-lactamase), Pm, Tm	32	Gm^s mutant of pSH9
pSH10	Gm, Km, Pc (β-lactamase), Pm, Tm	35	Strain SH10 (Table 1)
pSH11	Gm, Km, Tm	12	Strain SH11 (Table 1)

[a] Abbreviations: Cm, chloramphenicol; Em, erythromycin; Km, kanamycin; Pc, penicillin; and Tc, tetracycline.

Michael Reese Hospital into five classes based on phenotype, molecular weight, and restriction endonuclease digestion patterns (14). Resistance to gentamicin (Gm) and tobramycin (Tm) was mediated by these plasmids through the formation of 6'-aminoglycoside acetyltransferase and 2''-aminoglycoside phosphotransferase. In addition, classes 2 to 5 plasmids encoded 4'-aminoglycoside adenylyltransferase, an enzyme that mediated resistance to Tm and paromomycin (Pm). Class 3 plasmids also mediated resistance to ethidium bromide (EtBr), and classes 4 and 5 plasmids mediated resistance to benzylpenicillin through beta-lactamase production.

Media. Nutrient broth, brain heart infusion broth, and heart infusion broth were products of Difco Laboratories, Detroit, Mich. Tryptic soy broth was a product of BBL Microbiology Systems, Cockeysville, Md.

Plasmid transfer. Plasmids were transferred in a mixed culture by a method essentially like that of Lacey (15). Donor and recipient strains grown overnight at 37°C on brain heart infusion agar were subcultured to fresh medium. After 6 h at 37°C, cells were suspended in nutrient broth and adjusted by optical density to $\sim 10^7$ colony-forming units per ml. Donor and recipient suspensions (1.0 ml each) were added to 0.1 ml of a 0.2 M $CaCl_2$ solution and incubated at 37°C for 18 h with orbital shaking. Dilutions of the mixed cultures were plated on selective brain heart infusion agar plates to determine the number of resistant progeny and also the number of donor and recipient cells. We used 10 μg of Gm per ml as the selective agent and a combination of 10 μg of rifampin and 100 μg of streptomycin per ml for counterselection. This procedure uniformly gave no growth with separate cultures of the donor and recipient strains which were regularly made as controls. Transfer frequencies were expressed as the number of resistant progeny divided by the final donor count, except in studies of the cotransfer of plasmids, where the divisor was the final recipient count. In some experiments, we used 18 μg of EtBr per ml or 10 μg of Tm per ml to select for the transfer of appropriately resistant Gm^r plasmids.

For the selection of the transfer of other R-plasmids, we used erythromycin (6 μg/ml), chloramphenicol (12 μg/ml), or tetracycline (5 μg/ml).

Filter mating was initiated with brain heart infusion broth suspensions of donor and recipient cells (10^9 colony-forming units per ml) prepared from their 18-h growth on brain heart infusion agar. Equal portions (2.5 ml) of donor and recipient cells were mixed and filtered through a 25-mm nitrocellulose filter (0.45-μm pore diameter), which was then incubated on experimentally varied agar media for 18 h at 37°C. The resultant growth was suspended in 1 ml of the broth medium corresponding to the agar medium, diluted and plated on selective media.

Plasmid transfer by transduction was performed as previously described (3). The standard phage vector, φ11, was induced by UV light in *S. aureus* 8325 or in a derivative of RN450 previously lysogenized with φ11.

Analysis of plasmid DNA by restriction endonuclease digestion. Plasmid DNA was prepared by previously described methods (14). Restriction endonucleases *Hin*dIII, *Msp*I, *Xba*I, and *Eco*RI were purchased from New England Biolabs, Inc., Beverly, Mass., or Bethesda Research Laboratories, Inc., Gaithersburg, Md., and were used according to the manufacturer's instructions. Plasmid DNA fragments were analyzed by horizontal electrophoresis in 0.5 to 2.2% agarose gels. To aid in the physical mapping of plasmids, double digestions, using pairs of these restriction endonucleases, were performed. In addition, selected fragments were digested with a second restriction endonuclease (26). In this method, plasmid DNA was digested with a restriction endonuclease and subjected to electrophoresis in low-gelling-temperature agarose. Selected bands were cut out of the gel, melted at 60°C, digested with a second restriction endonuclease, and then analyzed by agarose gel electrophoresis.

Other methods. Susceptibility to antibiotics and to EtBr by disk diffusion methods was tested as previously described (13, 14). For testing for resistance to Pm, we used paper disks containing 10 μg of Pm. Assays for aminoglycoside-modifying enzymes were performed on crude staphylococcal extracts by phosphocellulose paper-binding procedures (11).

RESULTS

Plasmid transfer. In prior work, we transferred staphylococcal Gm^r plasmids by incubating mixed donor and recipient cells at high concentrations in a yeast extract, glucose, vitamins, and salts medium (37), together with added Ca^{2+} and lysozyme (13). Looking for a procedure using more nearly physiological conditions, we found that some of our Gm^r plasmids could be transferred readily by Lacey's simpler mixed-cell culture method (15, 16). When we used nutrient broth with added 0.01 M Ca^{2+} as the transfer medium, this procedure resulted in the transfer of Gm^r plasmids from many of our clinical isolates of *S. aureus* to derivatives of *S. aureus* RN450 and, to a lesser extent, to the apparently plasmid-free *S. epidermidis* SH29. In kinetic experiments with the transfer of pSH8 and pSH9 from their native hosts to a rifampin- and streptomycin-resistant derivative of RN450 (φ11), plasmid transfer was first detectable after 2 to 3 h of incubation. The number of Gm^r recipient staphylococci increased for about 12 h and thereafter remained constant for a total of 24 h. We obtained only a sporadic transfer of Gm^r plasmids without added Ca^{2+}. In contrast to Lacey's results, we found that Mg^{2+} (0.001 to 0.03 M) did not substitute for Ca^{2+}. Transfer frequencies were two to three orders of magnitude lower when we used heart infusion, brain heart infusion, or tryptic soy broth instead of nutrient broth as the transfer medium. We observed that staphylococci cultivated in nutrient broth with added 0.01 M Ca^{2+} exhibited large clumps within 2 to 4 h of incubation to a notably greater extent than they did after growth in nutrient broth without added Ca^{2+}. After these preliminary studies, all transfer experiments in liquid medium were performed in nutrient broth with added 0.01 M Ca^{2+} and an incubation time of 18 h.

TABLE 3. Mixed-culture transfer of Gm^r plasmids

Plasmid[a]	Transfer frequency[b] to:	
	S. aureus SH25	*S. epidermidis* SH29
pSH6	2.1×10^{-6}	$<5 \times 10^{-9}$
pSH8	4.7×10^{-5}	1.7×10^{-6}
pSH9	1.2×10^{-5}	1.4×10^{-7}

[a] Borne by donor *S. aureus* SH23.
[b] Resistant progeny count/final count.

To ascertain the transferability of Gm^r plasmids from a common donor strain, we first transferred representatives of our various classes of Gm^r plasmids from their wild-type hosts to SH23 by mixed culture in nutrient broth. The Gm^r progeny were shown to contain single plasmids with properties as previously described (14). They were then used as donor strains in transfer by mixed-cell cultures to SH25 or SH29. Plasmid pSH6, with a molecular mass of 12 megadaltons (Md), transferred to the *S. aureus* but not to the *S. epidermidis* recipient. In contrast, pSH8 and pSH9, with molecular masses of 30 and 38 Md, respectively, transferred intraspecifically at frequencies 5 to 20 times greater than did pSH6 and transferred interspecifically at lower but readily detectable rates (Table 3). The transfer frequencies of these plasmids from their wild-type hosts to these recipient strains were not significantly different from those of SH23. In other interspecific transfer experiments with SH29(pSH8), SH29(pSH9), SH29(pSH6), or *S. epidermidis* SH11 as the plasmid donor strains and an *S. aureus* recipient strain, pSH8 and pSH9 transferred interspecifically, but pSH6 and pSH11 did not. For this experiment, we obtained the SH29(pSH6) donor strain by our earlier lysozyme-mixed-culture method (13). Plasmids pSH7 and pSH10 had transfer properties similar to those of pSH8 and pSH9, in contrast to pSH6 and pSH11 (data not shown).

Since the donor strains in the foregoing experiments were lysogenic, their plasmids might have been transferred by spontaneous transduction. However, the transduction of plasmids as large as pSH9 has not been reported in *S. aureus*. Furthermore, in transduction experiments performed with the induction of phage φ11, we were unable to detect the transduction of pSH9 or pSH10 from SH23, whereas we had no difficulty in transducing the smaller plasmids pSH6 and pSH8. Presumably, the inability to transduce pSH9 was due to the fact that its DNA content was greater than that of the ~30-Md genome of the φ11 transducing phage (19). These results cast doubt on transduction as the mechanism of transfer of pSH9 in mixed culture and suggested the presence of another transfer mechanism for this and other staphylococcal Gm^r plasmids.

To evaluate the role of bacteriophage in the transfer process, we compared the transfer properties of some staphylococcal plasmids in a nonlysogenic system. Using phage φ11 induced by UV light, we transduced plasmids pSH6, pSH8, pI258, pC22.1, and pSH5 from SH25 to the nonlysogenic RN450. The multiplicity of phage to recipient was 0.01. Transductant strains that remained fully sensitive to the φ11 transducing phage were used as the donors in transfer experiments by mixed culture, with the nonlysogenic strain SH24 and the *S. epidermidis* strain SH29 as the recipients. pSH8 transferred to both recipients with frequencies as high as those from the lysogenic donor, but the transfer of the other plasmids was not detected. Eleven other independently derived transductants bearing pSH8 possessed similar transfer properties. Other evidence that the donor strains were not lysogenic included their failure to be lysed after exposure to UV light or mitomycin C (0.1 or 1.0 μg/ml) and the failure of the filtrates of their broth cultures to show lytic activity against RN450 or *S. aureus* 80CR5, which is uniformly sensitive to *S. aureus* typing phages (7). The transfer of pSH8 was not detected in experiments with cell-free filtrates of the donor strain. Transformation did not appear to be a plausible mechanism of transfer since it was not inhibited by the addition of bovine pancreatic DNase I (100 μg/ml) or micrococcal DNase (10 μg/ml) to the mixed cultures.

Each of the nonlysogenic donor strains was separately lysogenized with phage φ11, and the transfer experiments were repeated, using SH25 and SH29 as the recipients (Table 4). The transfer frequencies of pSH8 were not appreciably altered by the lysogenization. However, there was now a detectable intraspecific transfer of each of the other plasmids, presumably by spontaneous transduction, but no interspecific transfer occurred.

To obtain the nontransducible plasmid pSH9

TABLE 4. Effect of lysogeny with phage φ11 on plasmid transfer in mixed culture

Plasmid[a]	Transfer frequency[b] to:	
	S. aureus SH25	*S. epidermidis* SH29
pSH8	1.8×10^{-4}	1.5×10^{-6}
pSH6	2.8×10^{-6}	$<5 \times 10^{-9}$
pI258	4.9×10^{-6}	$<5 \times 10^{-9}$
pC22.1	5.3×10^{-8}	$<5 \times 10^{-9}$
pSH5	9.4×10^{-7}	$<5 \times 10^{-9}$

[a] Donor *S. aureus* RN450, bearing each of the listed plasmids, was lysogenized with phage φ11.
[b] Resistant progeny count/final donor count.

TABLE 5. Mobilization and cotransfer of plasmids in mixed culture

Plasmids[a]	Selective agent[b]	Transfer frequency[c] to *S. aureus* SH24	Cotransfer[d] of unselected plasmid
pSH5 and pSH8	Gm	1.8×10^{-4}	12/1,048
	Tc	8.7×10^{-6}	453/998
pC22.1 and pSH8	Gm	1.4×10^{-4}	382/1,150
	Cm	1.1×10^{-4}	309/887
pI258 and pSH8	Gm	1.0×10^{-4}	0/809
	Em	3.1×10^{-9}	1/1

[a] Borne by donor *S. aureus* RN450.
[b] Abbreviations: Tc, Tetracycline; Em, erythromycin.
[c] Resistant progeny count/final recipient count.
[d] Doubly resistant progeny count/total progeny count.

in a nonlysogenic host, we transferred it by mixed culture from lysogenic host strain SH25 to the nonlysogenic recipient SH22. Progeny colonies were screened for lysogeny by cross-streaking with RN450. We detected seven apparently nonlysogenic colonies that exhibited all of the resistances determined by pSH9. From one of these strains, pSH9 transferred in mixed culture intra- and interspecifically at frequencies similar to those seen in the lysogenic system (data not shown).

Both pSH8 and pSH9 could be serially transferred in mixed culture to other nonlysogenic *S. aureus* strains, namely 80CR5 and derivatives of RN450, and to *S. epidermidis* SH29.

These experiments suggested that some Gm^r plasmids transferred in mixed culture by a mechanism that was distinct from transduction or transformation, was not bacteriophage dependent, and that required cell-to-cell contact, i.e., by a conjugation-like mechanism. Other Gm^r plasmids, represented by pSH6 and pSH11, did not appear to possess this transferability trait.

We observed a similar transfer of Gm^r plasmids from 34 strains of *S. aureus* and 8 strains of *S. epidermidis* isolated at Michael Reese or at other hospitals in Chicago, New York, Los Angeles, and England. The resistance patterns mediated by the transferred plasmids were either Gm^r Pm^r Tm^r; $EtBr^r$ Gm^r Pm^r Tm^r; Gm^r Pm^r Tm^r penicillin resistant; or in one instance, $EtBr^r$ Gm^r Pm^r Tm^r penicillin resistant owing to penicillinase production.

Transfer of Gm^r plasmids by filter-mating. pSH8 transferred readily by filter-mating from its wild-type host to strain SH25. In contrast to the results of mating in broth, pSH8 transferred at similar frequencies (1.2×10^{-6} or 1.8×10^{-6}) whether nutrient agar or brain heart infusion agar was used as the medium for filter-mating. The addition of Ca^{2+} did not appreciably alter the frequency of transfer, nor was it inhibited by the chelation of Ca^{2+} by the addition of 0.02 M sodium citrate to the agar medium. We obtained comparable results with nonlysogenic derivatives of RN450 as the donor and recipient strains (data not shown). Other Gm^r plasmids that were transferable in 0.01 M Ca^{2+} nutrient broth also transferred by filter-mating in a nonlysogenic system, whereas pSH6 did not. These results added evidence that close cellular contact is required for the transfer of Gm^r plasmids and supported the notion that the effect of Ca^{2+} in broth mating is to enhance such contact.

Plasmid mobilization and cotransfer. To obtain plasmid heterodiploid strains, we first transduced pSH5, pC22.1, and pI258 separately into SH22. We then introduced pSH8 into each of these strains by transduction or by mixed culture with SH23(pSH8). At each plasmid transfer, we took care to choose nonlysogenic progeny by the tests listed above. The resultant plasmid heterodiploid strains were used as the donors in a nonlysogenic system with SH24 as the recipient and with selection for the transfer of each plasmid separately (Table 5). Screening for the unselected cotransfer of the second plasmid was performed by replica plating. With selection for Gm^r, pSH8 transferred at the same high frequency from each strain; pC22.1 cotransferred at relatively high frequency; and pSH5 cotransferred at lower frequency. Selection for resistance to tetracycline showed that pSH5 was mobilized to transfer independently of pSH8 and that pSH8 cotransferred at ~45% of the rate of pSH5. Selection for chloramphenicol resistance gave evidence of a similar mobilization of pC22.1 and cotransfer of pSH8. pI258 was neither mobilized nor cotransferred to any significant degree. In serial transfer experiments, the cotransferred plasmid markers continued to segregate at unchanged rates, indicating that no stable covalent linkage of the plasmids was present. Occasionally, we isolated strains that stably expressed some determinants of each parental plasmid; presumably, these reflected recombinant plasmids.

We looked for the possible cotransfer by pSH8 of methicillin resistance, a chromosomal marker in *S. aureus* (32). Using as donors three wild-type methicillin-resistant strains bearing transferred copies of pSH8, we did not succeed in transferring methicillin resistance.

Deletion mutants of Gm^r plasmids. A feature of the transfer of pSH8 and other Gm^r plasmids by mixed culture was the frequent, stable loss of unselected markers. Most experiments were performed with pSH8, which gave such mutations in ~1% of progeny. Restriction endonucle-

TABLE 6. Phenotypes of deletion mutants of pSH8

Phenotypic marker				No. of plasmids analyzed by restriction endonuclease digestion[a]
Gm	EtBr	Tra	Pm	
−	+	+	+	6
+	−	+	+	3
−	−	+	+	1
−	−	−	+	2
+	−	−	−	7
+	+	−	−	1
+	+	+	−	2

[a] The plasmids were derived from genetically independent deletions.

ase analysis showed that virtually all of these mutants represented deletions. Three methods were used to isolate a library of deletion mutants.

(i) Screening for loss of EtBr^r. We noted that EtBr-susceptible staphylococci grown on plates containing low concentrations of EtBr (0.5 to 2 μg/ml) were colored pink and fluoresced orange under UV light, whereas EtBr^r strains were unaltered in color and did not fluoresce. Presumably EtBr^r was conferred by a mechanism of exclusion. For the detection of deletion mutants, we transferred pSH8 into SH25 on plates containing EtBr (0.5 μg/ml), Tm (10 μg/ml), streptomycin (100 μg/ml), and rifampin (10 μg/ml). Colonies that exhibited orange fluorescence under UV light were screened for the loss of resistance to Gm, EtBr, or Pm and for the loss of transferability.

(ii) Replica plating. We transferred pSH8 into SH23, selecting for the transfer of Gm^r, Pm^r, or EtBr^r, and replicated the progeny to plates containing each of the unselected markers. Colonies that exhibited the loss of a resistance marker were also tested for transferability.

(iii) Positive selection for loss of Pm^r. We were unable to detect the loss of the Pm^r function of pSH8 by replica plating. For this purpose, we adapted a published method for positive selection for loss of resistance to kanamycin in *Escherichia coli* (33). Strain SH26, a streptomycin-dependent, rifampin-resistant mutant of SH21, was able to grow on media containing Pm instead of streptomycin. However, strain SH26(pSH8) did not grow in the presence of Pm, owing to the inactivation of Pm by the 4′-adenylyltransferase encoded by pSH8. The same host strain containing plasmids that did not encode adenylyltransferase activity grew on Pm plates. Pm lacks the functional groups attacked by the other aminoglycoside-modifying enzymes encoded by the Gm^r plasmids. Therefore, the growth of SH26(pSH8) on Pm plates could be utilized to positively select for pSH8 mutants that lost 4′-adenylyltransferase activity. To this end, we transferred pSH8 from SH23 into SH26, using plates containing 10 μg of rifampin per ml, 10 μg of Pm per ml, and selective concentrations of either EtBr or Gm. We confirmed that the SH26 transconjugants no longer mediated Pm^r by transferring their plasmids to a streptomycin-independent derivative of RN450 and testing the resulting colonies for the markers of pSH8. All were susceptible to Pm. Among these, two clones exhibited a loss of Pm^r only, and two others exhibited a loss of Pm^r and transferability, phenotypes which we had not obtained by other methods. The assay of one of these mutants confirmed that it had lost 4′-adenylyltransferase activity.

Analysis and physical mapping of pSH8 and its deletion mutants. The phenotypic analysis of the deletion mutants of pSH8 provided evidence of the genetic structure of the wild-type plasmid. We scored the drug resistance and transfer properties of all of our pSH8 deletion mutants. In the nonlysogenic system, the mutant plasmids proved to be either fully self-transferable, like the parental plasmid, or nontransferable (Tra$^-$), like pSH6. However, when the host strain and recipient were lysogenized with φ11, the Tra$^-$ mutants transferred at rates comparable to that of pSH6, presumably due to spontaneous transduction (data not shown). Table 6 lists the mutant phenotypes of pSH8 isolated. Assuming that a single genetic event was responsible for each altered phenotype, these data suggested the following genetic linkage: Gm, EtBr, Tra, Pm.

By means of transduction with φ11, we made plasmid heterodiploid strains comparable to those in Table 5, but containing deletion mutants of pSH8 instead of the wild-type plasmid. Two mutants were still Tra, but one had lost Gm^r, and the other had lost Gm^r and EtBr^r. Two other independent mutants were Tra$^-$ and had lost both Gm^r and EtBr^r. A third Tra$^-$ mutant had lost EtBr^r and Pm^r. In filter-mating experiments, we selected for the transfer of the plasmids from the heterodiploids either with 100 μg of kanamycin per ml (to which all of the mutant pSH8 plasmids employed still mediated resistance) or with tetracycline or chloramphenicol as previously described. The Tra$^-$ mutant plasmids failed to mediate the transfer of resistance to kanamycin, tetracycline, or chloramphenicol, whereas the mutant plasmids that were still Tra gave results comparable to those shown with the wild-type pSH8 (Table 5). Thus, the loss of the Tra function eliminated not only the transfer of pSH8, but also the cotransfer and mobilization of the independent coresident R-plasmids.

*Hin*dIII digests of plasmid DNA from multiple independent deletions of pSH8 showed that, for

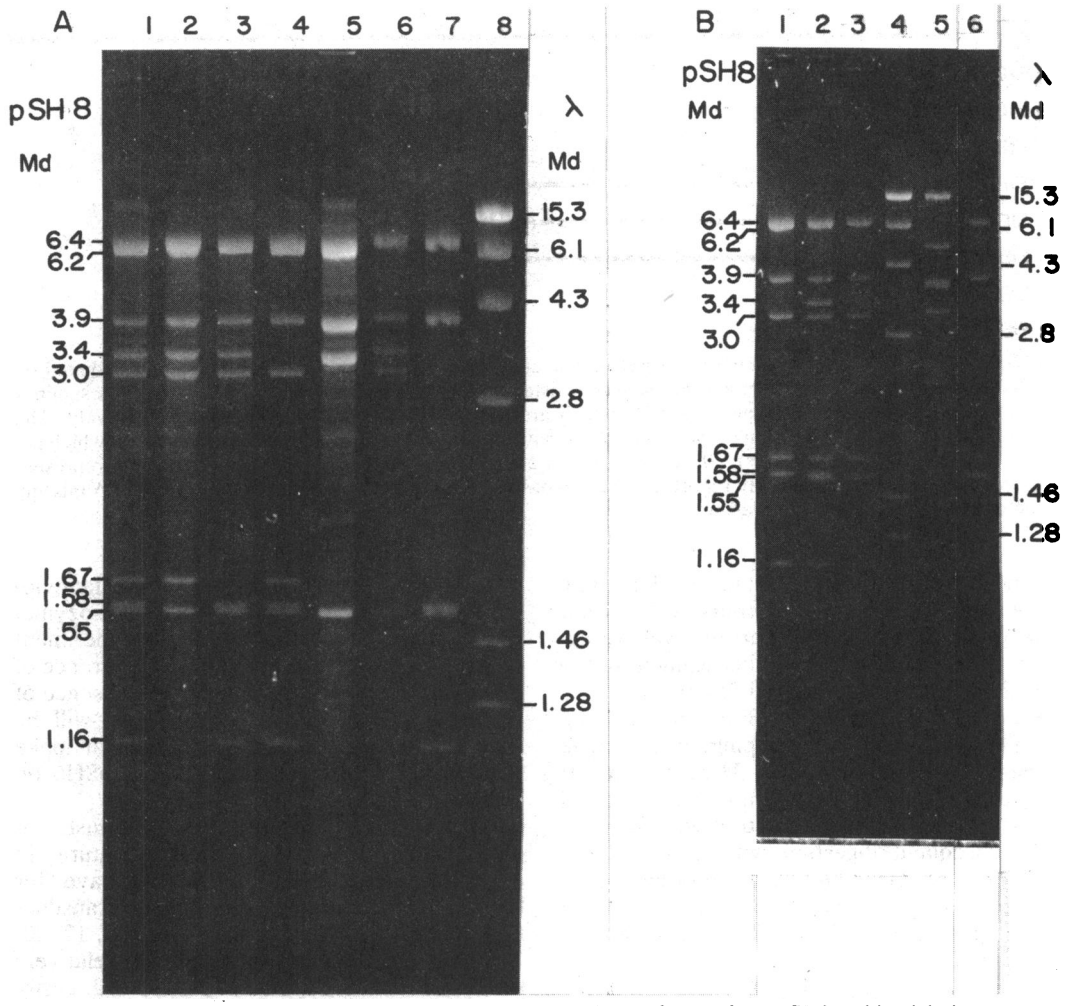

FIG. 1. Gel electrophoretograms of *Hin*dIII digestion products of DNA from pSH8 and its deletion mutants. The numerical lane headings denote DNA from plasmids that mediated the following phenotypes. (A) Lane 1, Gm, EtBr, Tra, Pm (wild-type pSH8); lane 2, Gm^s, EtBr, Tra, Pm; Lane 3, Gm, $EtBr^s$, Tra, Pm; lane 4, Gm, EtBr, Tra, Pm^s; lane 5, Gm^s, $EtBr^s$, Tra^-, Pm; lane 6, Gm^s, $EtBr^s$, Tra, Pm; lane 7, Gm, $EtBr^s$, Tra^-, Pm^s; and lane 8, phage λ. (B) Lane 1, Gm, EtBr, Tra, Pm^s; lane 2, Gm, EtBr, Tra, Pm (wild-type pSH8); lane 3, Gm, EtBr, Tra, Pm^s; lane 4, phage λ; lane 5, phage λ digested by *Eco*RI; and lane 6, Gm, EtBr, Tra^-, Pm^s. The numbers to the left of each panel signify the molecular masses of the *Hin*dIII fragments of wild-type pSH8. Those to the right of each panel list the molecular masses of the *Hin*dIII fragments of phage λ. Lane 4 (A) and lane 3 (B) represent duplicate digestions of DNA from the same mutant plasmid. Electrophoresis was performed for 18 h in 1% agarose at 60 V. Not shown are small fragments from *Hin*dIII digests of plasmid DNA, detected by electrophoresis in 2.2% agarose, as follows: (A) lanes 5 and 6, one fragment of 0.22 Md; and lane 7, one fragment of 0.22 Md and one fragment of 0.44 Md.

each mutant phenotype, the plasmid digestion pattern was distinctive. Plasmids with corresponding mutant phenotypes had very similar or identical digestion patterns. Representative examples are shown in Fig. 1. Evidently, there were highly favored sites for the pSH8 deletions. Furthermore, these sites were near or at the loci of *Hin*dIII scissions, since the deleted plasmids produced either no new *Hin*dIII fragments or only one or two additional fragments of 0.2 or 0.4 Md.

These digests, illustrated diagrammatically in Fig. 2, showed that the Tra phenotype was specifically associated with *Hin*dIII fragments 1 and 5. Likewise, $EtBr^r$ was associated with *Hin*dIII fragment 6. The loss of resistance to Gm and Pm was associated with the loss of specific segments of DNA, but we could not determine

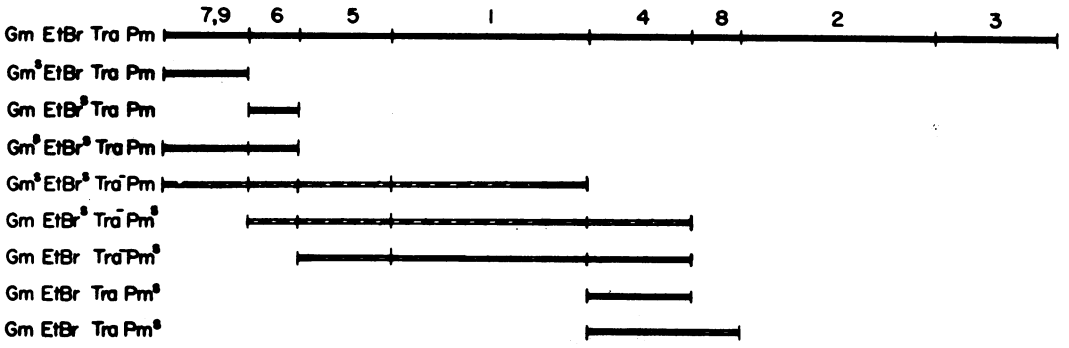

FIG. 2. Diagram of deletion mutants of pSH8. The alphabetic symbols denote the phenotypes mediated by pSH8 and its mutants. Gm, EtBr, and Pm signify resistance to Gm, EtBr, and Pm. The superscript s signifies susceptibility. Tra and Tra$^-$ signify plasmids which are transferable and nontransferable, respectively. The numerals denote *Hin*dIII digestion fragments of pSH8 ranked in order of decreasing molecular masses, which are (i) 6.4, (ii) 6.2, (iii) 3.9, (iv) 3.4, (v) 3.0, (vi) 1.67, (vii) 1.58, (viii) 1.55, and (ix) 1.16 Md. These values represent slight revisions of those previously reported (14). The dashed bars indicate the segments of pSH8 DNA missing in the deletion mutants of that plasmid.

whether these segments contained all or only part of the coding for these traits. We did not isolate derivatives that lost part of *Hin*dIII fragments 2 and 3. Presumably, this segment of the plasmid encodes vital plasmid functions.

We extended the analysis of the physical map of pSH8 by restriction mapping, using single digests with *Hin*dIII, *Eco*RI, *Msp*I, and *Xba*I; double digests with pairs of these enzymes; and the isolation of selected restriction fragments with subsequent digestion with a second enzyme. The results established the order shown in Fig. 3, with residual uncertainty about the relative positions of *Hin*dIII fragments 7 and 9.

DISCUSSION

In this paper, we have presented evidence that staphylococcal Gm^r plasmid pSH8 and other plasmids encode their own transfer intra- and interspecifically by a conjugation-like mechanism which, in pSH8, is mediated by a specific segment of the plasmid genome. This interpretation is supported by the ability of pSH8 to mobilize the independent transfer of otherwise nontransferable plasmids and by the loss of this mobilizing function in the Tra$^-$ mutants of pSH8.

The failure of pSH6 or pSH11 to transfer between nonlysogenic staphylococci would appear to be discordant with our previous report of the intra- and interspecific transfer of these plasmids in vitro and on human skin (13). However, our earlier interspecific transfer of pSH6 and pSH11 in vitro was based upon the incubation of donor and recipient in a medium containing lysozyme. In the absence of lysozyme, we detected no transfer in vitro. We confirmed these results in our current studies. Interspecific transfer on skin was performed with staphylococci suspended in 1 M NaCl without lysozyme. The simultaneous repetition of this experiment on skin and in vitro confirmed the occurrence of interspecific transfer on skin and the absence of such transfer in vitro. Further study will be necessary to determine the factors that make possible the interspecific transfer of pSH6 on skin.

Previous studies by others of the transfer of staphylococcal plasmids in mixed culture, in experimental infections, and on skin have, for the most part, implicated spontaneous transduction as the mechanism of transfer (7, 8, 17, 20, 22, 25, 39). The plasmids have been relatively small, with DNA contents of 3 to 23 Md, easily accommodated within the head of the staphylococcal transducing phage. However, transduction, for example, by some undetected defective phage, could not readily account for the transfer of a plasmid such as pSH9, which is appreciably larger than the ~30-Md genome size of ϕ11, the transducing phage used in our experiments, nor for the interspecific transfer of plasmids between *S. aureus* and *S. epidermidis*.

There are reports by others of R-plasmid transfer in staphylococci that suggest a conjugal mechanism. Witte transferred R-plasmids between strains of *S. aureus* and between *S. aureus* and *S. epidermidis* by the incubation of dense suspensions of these bacteria, as much as 10^{11} bacteria per ml (37–39). He adduced evidence pointing to a conjugal mechanism of transfer, including the observation that plasmid transfer was enhanced by the cell-fusing agents oleic acid or polyethylene glycol. The physiological significance of his experiments may be limited by the fact that a density of ~10^{10} bacteria per ml was required for detectable transfer. Naidoo

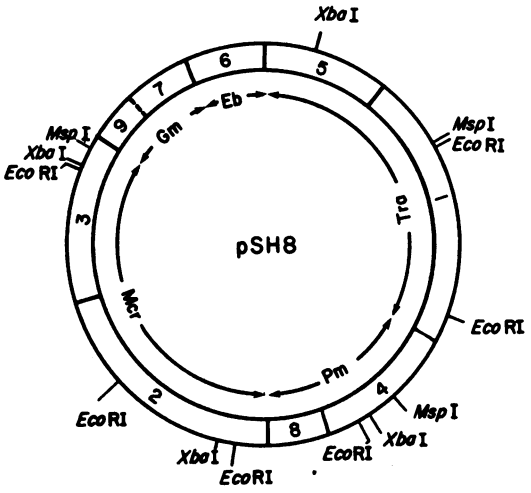

FIG. 3. Physical and genetic map of pSH8. The numbers in the outer ring designate the *Hin*dIII segments in order of decreasing mass. The relative positions of fragments 7 and 9 are indeterminate. Gm, Eb, and Pm designate the plasmid DNA segments that mediate resistance to Gm, EtBr, and Pm, respectively. Tra represents the DNA segment that mediates self-transferability in mixed culture. Mcr represents the DNA segment which is believed to mediate plasmid maintenance, compatibility, and replication.

and Noble transferred a Gm^r plasmid by mixed culture or filter-mating from a clinical isolate of *Staphylococcus hominis* to several different *S. aureus* strains (23). The recipients, in turn, transferred the plasmid to the original *S. hominis* host and to other *S. aureus* strains. Culture supernatants and mitomycin C-induced lysates of the *S. hominis* strain did not transfer Gm^r. DNase reduced transfer frequency fivefold. In our earlier experiments with lysozyme-aided transfer, we found that DNase inhibited Gm^r-plasmid transfer, but we were not able to duplicate this observation with the mixed-cell culture method used in the current study (13). Otherwise, our results and those of Naidoo and Noble were generally comparable.

Staphylococcal plasmids have been transferred experimentally by the transformation or fusion of protoplasts (9, 18). There is little evidence as yet that these mechanisms are active in mixed culture.

The conjugal transfer of plasmids has been studied extensively in *Streptococcus* sp., another gram-positive coccal genus (1). Clewell showed that some recipient *Streptococcus faecalis* strains secrete one or more soluble pheromones into their culture medium (6). These agents, in some unknown way, induced a plasmid-dependent process that caused the appearance of a proteinaceous material on the donor's surface and thereby facilitated the clumping of donor and recipient, with subsequent plasmid transfer. The possibility that a system similar to that in *Streptococcus* sp. also operates in staphylococci is heightened by the observation that plasmids which mediated resistance to macrolides, lincomycin, and group B streptogramins were transferable from streptococcal species to *S. aureus* and, subsequently, between strains of staphylococci (7, 30). Moreover, the apparent identity, reported by others, between plasmid-borne erythromycin resistance determinants in *S. faecalis* and *S. aureus* suggests that there may be a mechanism for genetic transfer between these genera, either directly or through some intermediary (36). Similar observations were made in these species by Courvalin et al. for aminoglycoside resistance determined by 3′-phosphotransferase (4, 5). Conjugation, a relatively promiscuous process, would appear to be a better candidate than transduction for genetic exchange between genera.

Irrespective of the precise mechanism, the demonstrable transfer of Gm^r plasmids from staphylococci isolated at our hospital and elsewhere provides an attractive model for the in vivo dissemination of these types of Gm^r plasmids both intra- and interspecifically. Furthermore, their ability to mobilize other plasmids may contribute to the spread of resistance determinants other than those encoded by the Gm^r plasmids.

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